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Experimental methodology to quantify *Candida albicans* cell surface hydrophobicity

Mariana Henriques, Kelly Gasparetto, Joana Azeredo & Rosário Oliveira*
Centro de Engenharia Biológica – CEB, Universidade do Minho, Campus Gualtar, 4710-057 Braga, Portugal
**Author for correspondence (Fax: +351 253678986; E-mail: roliveira@deb.uminho.pt)*

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Abstract

A new method of formation of yeast cell lawns for contact angle measurement (with water, formamide and 1-bromonaphthalene) is described. The cell lawns were formed on agar layers avoiding liquid penetration. The method was validated by comparing the hydrophobicity of *Candida albicans* grown at different temperatures and the hydrophobicity of bacterial cell lawns built on agar layers and obtained by the usual filtration method.

Introduction

Candida albicans is an opportunistic fungal pathogen that may be present as a normal component of the body microflora. It is responsible for a variety of diseases especially in immunocompromised and immunosupressed hosts (Cotter & Kavanagh 2000).

The ability of microorganisms to adhere to surfaces exposed to the flushing action of fluids is a prerequisite for successful colonization. Therefore microbial adherence is an essential first step in the development of infection (Waters *et al.* 1997).

The yeast surface is the site of physical-chemical interactions with the host tissues leading to its adherence (Cannon & Chaffin 1999). Previous studies of *C. albicans* cell wall have suggested a relationship between cell surface hydrophobicity (CSH) and adherence capability (Panagoda *et al*. 1998). The CSH of yeast cells is affected by various factors, including temperature, nutrition and stage of growth (Fukazawa & Kagaya 1997). One of the most significant events in the pathogenesis of candidosis is the ability of commensal *C. albicans* to switch from a hydrophilic to a hydrophobic state (Hazen & Hazen 1992).

There are a few methods to determine hydrophobicity: microbial adhesion to hydrocarbons, such as xylene (Samaranayke *et al.* 1995) and hexadecane (Rodrigues *et al.* 1999); salt aggregation (Lindahl *et al.* 1981); hydrophobic interaction chromatography (Sklodowska & Matlakowska 1998); hydrophobic microsphere assay (Hazen & Hazen 1987, Hazen *et al.* 2001); coaggregation with *Fusobacterium nucleatum* (Jabra-Rizk *et al.* 2001) and contact angles (van der Mei *et al.* 1998). The most common methods used to determine *Candida albicans* hydrophobicity are microbial adhesion to hydrocarbons and hydrophobic microsphere assay, but these methods only give qualitative information, because they are based on the microorganism affinity for a polar and an apolar phase or a ligand.

The measurement of contact angles, formed by sessile drops of three different liquids (two polar and one apolar), enables the calculation of the surface free energy and the degree of hydrophobicity (van Oss 1997). This method has been largely used in bacterial cell surface characterization, by measuring the contact angles formed on cell lawns obtained on membrane filters (Absolom *et al.* 1983). However, as yeasts are concerned the building of cell lawns by filtration leads to a very porous mat, on account of the yeast cells dimensions, promoting the immediately sucking of the liquid drop. This is why it is much more common to characterize yeast hydrophobicity by hydrophobic microsphere assay or microbial adhesion to hydrocarbons. Thus, the aim of this work was to develop a

methodology to overcome the experimental drawback of contact angle measurement on yeast cells surfaces.

Materials and methods

Yeast growing conditions

Candida albicans cells were sub-cultured in Sabouraud dextrose agar for 24 h and then grown in Sabouraud dextrose broth for 18 h, until the stationary phase, at 23 °C and 37 °C under agitation.

Preparation of samples

Cells were harvested by centrifugation at 2600 *g* for 10 min and washed with increasing concentrations of ethanol in water (10, 20 and 50% v/v). The resulting pellet was c.f.u. resuspended in 50% (v/v) ethanol to give 10^9 ml.

A solution of 20 g agar l^{-1} and 10% (v/v) glycerol was cast onto a microscope slide (75 × 25 mm). Two ml of the yeast suspension was spread over the solidified agar layer to cover the entire surface. This layer was allowed to dry at room temperature (for 10–15 h), and two more layers were added, with drying between them.

Contact angle measurement

Contact angles were measured by the sessile drop technique, on the cell lawns prepared previously, using an apparatus model OCA 15 PLUS, DATAPHYSICS.

The measurements were performed at room temperature, using three different liquids: water, formamide and 1-bromonaphthalene. Every assay was performed in triplicate and at least 10 contact angles, per sample, were measured.

Results and discussion

A pre-requisite for successful contact angle measurement is the uniformity and homogeneity of the surface. Thus, it was important to guarantee that the cells were spread uniformly along the agar layer. This was confirmed by microscope inspection, which also confirmed that the slide was completely covered. Moreover, the small standard deviation of the contact angle values, measured in different points of a slide (Table 1), is evidence of the homogeneity of the cell lawn.

Table 1. Average and standard deviation (stand dev) of 10 measurements of contact angles of water, formamide and l-bromonaphthalene, on *C. albicans* lawn.

	Contact angle (°)		
	Water	Formamide	l-Bromonaphthalene
Average	40	52	98
Stand dev	4	5	3

Table 2. Mean contact angles and respective standard deviation obtained, with water, formamide and l-bromonaphthalene, over a lawn of *C. albicans* after washing with ethanol or water.

Washing solute	Contact angle (°)		
	Water	Formamide	l-Bromonaphthalene
Water	37 ± 2	48 ± 2	98 ± 3
Ethanol	41 ± 4	53 ± 4	95 ± 5

From Table 1, it is clear that the error associated to the measurement does not exceed 10%. According to Doyle & Rosenberg (1990) the contact angle measurement technique is the method that presents a higher confidence level in determining the hydrophobicity.

To confirm that the contact angles were being measured on a cell layer rather than on the agar, contact angles of water were measured on the agar layer without cells. The average value of the water contact angle on the agar surface ($51 \pm 3°$) is higher than on the cell lawns ($40 \pm 4°$). This confirms that the solid agar acts only as a supporting surface for the cells.

The cells were washed with increasing concentrations of ethanol in order to reduce the dehydration time (normal procedure to prepare cells for SEM observation). However, it was necessary to determine if ethanol interferes in yeast cell surface properties. For that, an assay was performed using water instead of ethanol to wash the cells. Table 2 shows the average contact angles obtained after washing with ethanol or water.

Similar contact angles were obtained with the different cell washing procedures (ethanol or water). So, as the drying process is faster with ethanol and it does not interfere with the surface properties, it is more advantageous to use this washing procedure.

The relation between the contact angle (θ) formed by a liquid over a solid surface and the components of the surface tension (of the liquid-l and surface-s): apolar (γ^{LW}: Lifshtiz–van der Waals) and polar ($\gamma^{AB} =$

Table 3. Values of the components of surface tension of water, formamide and l-bromonaphthalene.

	Surface tension (mJ m^{-2})			
	γ_l^{tot}	γ_l^{LW}	γ_l^+	γ_l^-
Water	72.8	21.8	25.5	25.5
Formamide	58	39	2.3	39.6
l-Bromonaphthalene	44.4	44.4	0	0

$2 \times \sqrt{\gamma^+\gamma^-}$: Lewis acid-base) can be established by the Young–Good–Girifalco–Fowkes equation (van Oss *et al.* 1987).

$$(1+\cos\theta)\gamma_l = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^+\gamma_l^-} + \sqrt{\gamma_s^-\gamma_l^+}\right) \quad (1)$$

As this equation has three unknown surface parameters, three different liquids (two polar and one apolar) are needed to calculate the surface tension ($\gamma^{tot} = \gamma^{AB} + \gamma^{LW}$). It is important that the probe liquids have a surface tension higher than that of the solid sample to avoid the spreading of liquid on the surface. So, this parameter should be higher than 40 mJ m^{-2} (van Oss *et al.* 1987). Water and formamide were the polar liquids used and l-bromonaphthalene the apolar one. Their corresponding surface tensions are presented in Table 3.

According to van Oss (1997) the hydrophobicity of a given material (s) can be defined in terms of the variation of the free energy of interaction between two moieties of that material immersed in water (w). The free energy comprises a polar (AB) and an apolar (LW) component ($\Delta G_{sws}^{tot} = \Delta G_{sws}^{LW} + \Delta G_{sws}^{AB}$) and the variation of the total free energy is given by:

$$\Delta G_{sws}^{tot} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 - 4\left(\sqrt{\gamma_s^+\gamma_s^-} + \sqrt{\gamma_w^+\gamma_w^-} - \sqrt{\gamma_s^+\gamma_w^-} - \sqrt{\gamma_s^-\gamma_w^+}\right) \quad (2)$$

When the value of ΔG_{sws}^{tot} is negative (the free energy of interaction between molecules is attractive) it means that the cells have less affinity for water than among themselves, meaning that they have a hydrophobic character. On the contrary, cells are hydrophilic when this value is positive ($\Delta G_{sws}^{tot} > 0$).

Table 4. Values of the average contact angle and respective standard deviation obtained for *Candida albicans* grown at 23 °C and 37 °C.

	Contact angle (°)		
Growth temp (°)	Water	Formamide	l-Bromonaphthalene
23	41 ± 4	53 ± 4	95 ± 5
37	40 ± 4	49 ± 5	75 ± 7

Table 5. Values of the apolar (γ^{LW}) and polar (γ^{AB}, γ^+, γ^-) components and total surface tension (γ^{tot}) and the free energy of interaction between cells of *C. albicans* and water (ΔG_{sws}^{tot}) for cells grown at different temperatures.

	Surface tension (mJ m^{-2})					
Growth temp (°C)	γ^{LW}	γ^+	γ^-	γ^{AB}	γ^{tot}	ΔG_{sws}^{tot}
23	9.2	7	51.9	38.2	47.4	15.3
37	17.7	3.1	50.6	25	42.7	26.9

As stationary-phase *C. albicans* exhibits growth temperature-dependent expression of surface hydrophobicity. Cells were grown at 23 °C and 37 °C to compare their behaviour concerning CSH. Table 4 shows the values of the average contact angle of the three liquids used (water, formamide and l-bromonaphthalene) on *C. albicans* cell lawns.

The value of the water contact angle can give preliminary information on the hydrophobicity of the cells. If the value is over 50° the surface is considered hydrophobic; on the contrary, the surface is hydrophilic if the angle is lower than 50° (van Oss & Giese 1995). In this case both water contact angles are not very distant from 50°, which means that for both growth temperatures the cells do not have well defined hydrophobic character.

The values of the contact angles of the three liquids (Table 4) and Equations (1) and (2) allow the calculation of the surface tension components and the variation of the free energy of yeast cells when immersed in water (Table 5).

From Table 5 it can be seen that cells grown at 37 °C are more apolar (higher γ^{LW} value) than cells grown at 23 °C. So, they have less affinity to polar liquids, as can also be confirmed from water and formamide contact angles (Table 4). In both growing conditions the negative component of polar surface

tension (γ^-) is much higher than the positive (γ^+), eliciting the idea that both strains are predominantly electron donors. The total surface tension is higher for the cells grown at 23 °C. Although ΔG_{sws}^{tot} is positive in both cases, the degree of hydrophobicity is higher for cells grown at 23 °C than for cells grown at 37 °C (ΔG_{sws}^{tot}@23 °C < ΔG_{sws}^{tot}@37 °C).

The differences in the cell surfaces (presence of hydrophobic proteins, formation and size of germ tubes) are responsible for the differences in the hydrophobicity of cells grown at different temperatures. This was confirmed with several methods used to study *Candida albicans* CSH, such as hydrophobic microsphere assay (Hazen & Hazen 1992) and co-aggregation (Jabra-Rizk *et al.* 2001), which gave also different hydrophobicities for cells grown at 23 and 37 °C. However in these assays, cells of the bacterium *F. nucleatum* and microsphere beads were able to penetrate the spaced-out short fibrils of the fibrilar layer of hydrophobic cells and adhere to receptors embedded in the cell wall on the yeast. Thus the attachment is also based on specific interactions. Moreover, the hydrophobic microsphere assay gives the average CSH level for the cell population and therefore the CSH values for a hydrophobic population of cells at different stages of growth may vary.

The problem with these methods, and others like microbial adhesion to hydrocarbons, is that other forces (as electrostatic), besides hydrophobic ones may interfere in the interaction between the ligands and the cell surface, and so, surface hydrophobicity is being masked. Doyle (2000) analysed different methods to determine hydrophobicity and concluded that the contact angle method is probably the most definitive way to determine cell surface hydrophobicity, once it gives an average value of hydrophobicity and does not take into account cell cycle variations or individual cell contributions.

The contact angle technique is a method frequently used to evaluate bacterial cell surface hydrophobicity. In the traditional method, samples are prepared by filtrating a cell suspension forming a cell lawn over a membrane filter (Busscher *et al.* 1984). To validate the experimental technique proposed in this work, bacterial (*Pseudomonas aeruginosa*) cell surface hydrophobicity, calculated using the values of the contact angles formed on cell lawns prepared by filtration ($\Delta G_{sws}^{tot} = 17.5$ mJ m^{-2}), was compared with that obtained with contact angles formed on cell lawns built on agar ($\Delta G_{sws}^{tot} = 19.9$ mJ m^{-2}). The values of the degree of hydrophobicity are similar for both experimental techniques. This demonstrates that it is valid to apply the contact angle technique to bacterial cell lawns built on agar as well as in filtered layers. Thus, eliciting the application of the method for yeast cell surface hydrophobicity characterization.

Conclusions

The aim of this work was the development of an experimental technique to study quantitatively the hydrophobicity of *Candida albicans*. In this method yeast cell lawns were prepared on agar layers rather than by filtration, overcoming the experimental drawback of the traditional method. In order to prove that the method could be applied successfully it was confirmed that the agar layer and the washing procedure using ethanol do not interfere with the measurement and that the yeast cell layers were uniformly built. Moreover, the technique proposed was also compared with the traditional method using bacterial cell lawns and similar results were obtained.

Considering the total set of results, it can be said that the proposed method is reliable for quantifying the degree of yeast cell surface hydrophobicity.

As the growth temperature can influence the physico-chemical surface properties of yeast *Candida albicans*, to evaluate the hydrophobic properties the assays were performed with cells grown at 23 °C and 37 °C. Although the difference in the CSH is not very significant, it can be said that cells grown at 37 °C are less hydrophobic when compared with cells grown at 23°C.

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